Two Independent Genetic Origins of β+-Thalassemia Due to -31 A to G Mutation in Thai and Japanese Populations

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ABSTRACT Haplotype associated with the -31 (A-G) β+ -thalassemia gene in seven Thai individuals were examined and compared with that described originally in Japanese. Seven polymorphic restriction sites within β-globin gene cluster were determined using allele specific polymerase chain reaction (ASPCR) methods newly developed for rapid β-globin haplotyping. A concordant result of DNA polymorphisms examined using ASPCR and conventional PCR-restriction fragment length polymorphism (PCR-RFLP) method was observed. It was found that all these seven Thai β+-thalassemia alleles were associated with the β-globin haplotype (+ - - - - - +), which is different from that described for a Japanese subject (- + + - + + -). This indicates two independent origins. As compared to the PCR-RFLP method, β-globin haplotyping using ASPCR developed is easier, rapid, less time-consuming and requires no restriction digestion. The methods should also prove useful in population genetic study and linkage analysis of β-hemoglobinopathy.

INTRODUCTION

Beta thalassemia is a group of β-globin gene disorders resulting from defects in the β-globin chain production. It is very prevalent among Southeast Asian populations and the Chinese but rarely found among northern Asians including Japanese and Korean. There are two main types. These are a β0-thalassemia when there is an absence of β-globin chain synthesis and a β+-thalassemia when there is a reduction in β-globin production (Higgs et al. 2012; Thein 2013; Williams and Weatherall 2012). While several hundreds of β-thalassemia mutations have been identified among world populations, each ethnic group has been shown to have several distinct mutations. For example, approximately 20 different mutations including both β0- and β+-thalassemia have been described in northeast Thailand. Of these, β+-thalassemia constituted

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26.7 percent of all β thalassemia alleles observed (Yamsri et al. 2010). Though with relatively much lower prevalence, as many as 47 different β-thalassemia mutations have been found among Japanese, and this data indicates the diverse heterogeneity of this disease in Japan. Among the mutations observed, a-31 A-G mutation within the TATA box was the most common one. This -31 A-G mutation has been thought to be unique to Japanese and has been arisen after Japan islands were separated from the Asian continent (Hattori et al. 2003; Yamashiro and Hattori 2015). Although this mutation has been rarely described in other populations, a sporadic case of Thai hemoglobin (Hb) E-β+-thalassemia patient with mild hypochromic microcytic anemia with this mutation has been documented (Vathana et al. 2005). It has recently been found that this -31 A-G mutation was the second most common β+- thalassemia allele among northeast Thai population (Yamsri et al. 2015). No ethnic relationship between Japanese and Thai has been noted. Haplotype analysis is used widely to determine genetic heterogeneity and origins of thalassemia and abnormal Hb genes in human populations (Mahdavi et al. 2015; Singha et al. 2015; Zhang et al. 2015).

Objectives of the Study

In order to address the genetic origin of this -31 A-G mutation in Thai population, the β-globin gene haplotype associated with this mutation in Thai subjects has been investigated and results compared with that of a Japanese patient previously documented (Takihara et al. 1986). Rapid β-globin gene haplotyping based on allele specific PCR developed was described.

MATERIALS AND METHODS

Specimens

Ethical approval of the study protocol was obtained from the Institutional Review Board of Khon Kaen University, Thailand (HE572237). Archival DNA specimens of Thai subjects with the -31 A-G mutation β$^+$-thalassemia were obtained from the earlier study at the thalassemia service unit of the Centre for Research and Development of Medical Diagnostic Laboratories, Khon Kaen University, Khon Kaen, Thailand (Yamsri et al. 2015). Data was finally summarized from seven unrelated subjects whose β-globin gene haplotypes could be accurately segregated.

Analysis of DNA Polymorphisms within the β-Globin Gene Cluster using Allele Specific PCR

Seven DNA polymorphisms within the β-globin gene cluster including ε-*Hinc* II, ^{G}γ- and ^{A}γ-*Hind* III, ψβ- and ψ3'yb-*Hinc* II, β-*Ava* II and β-*Hinf* I are routinely determined in the laboratory using PCR-RFLP assays as described (Fucharoen et al. 2002). In order to provide rapid analysis of these DNA polymorphisms, methods based on allele specific PCR were developed. Allele specific primers for these seven polymorphic sites are listed in Table 1 and depicted in Figure 1. The seven DNA polymorphisms were examined in three multiplex and a single monoplex PCR formats without restriction digestion. These PCR formats including multiplex PCRs for ε-*Hinc* II and ^{G}γ-*Hind* III), (^{A}γ-*Hind* III and 3ψβ-*Hinc* II, β-*Ava* II and β-*Hinf* I, and a monoplex PCR for (ψβ-*Hinc* II) were performed separately for the presence (using the plus primers) and absence (using the minus primers) of polymorphic sites. In each PCR reaction, additional primers generating an internal control fragment as shown in Figure 1 [either 1,068

bp with (SF7 and SF8), 578 bp with (γ$_4$ and γ$_5$) or 980 bp with (SF11 and SF 12)] were also added. PCR mixture (50 l) contains 100 ng genomic DNA, 15 pmole of each primer (either plus or minus specific primers), 200 mM dNTPs and 1 unit *Taq* DNA polymerase (New England Biolab Inc., MA, USA) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01 percent gelatin and 3 mM MgCl$_2$. The amplification reaction was carried out on a T-Personal Thermocycler (Biometra; GmbH, Gottingen, Germany). After initial heating at 95°C for 3 minutes, a 30 cycles PCR with 94°C, 1 minute - 66°C 1 minute 30 seconds, and a final extension step at 72°C, 10 minutes was carried out. The amplified PCR product was separated on 2.0 percent agarose gel electrophoresis and visualized under UV light after ethidium bromide staining.

RESULTS

The hematological characteristics of the seven Thai subjects with heterozygous β$^+$-thalassemia (-31 A-G mutation) are presented along with that described for a Japanese subject with homozygote for the same mutation in Table 2. Unlike the homozygous state described for a Japanese patient who had mark hypochromic microcytic anemia, all the Thai subjects with heterozygous β$^+$-thalassemia had normal Hb and Hct levels with slightly reduced MCV (76.8 ± 7.5 fL) and MCH (24.5 ± 1.0 pg) values. Hb analysis revealed as expected, elevation of Hb A$_2$ ($4.5 \pm 0.7\%$) and normal level of Hb F ($0.9 \pm 0.4\%$) in all cases.

Table 2: Hematological findings of the seven Thai subjects with heterozygous β$^+$ -thalassemia with -31 A-G mutation (mean±SD) as compared to that of a Japanese patient with homozygous for the same mutation reported previously (Takihara et al. 1986)

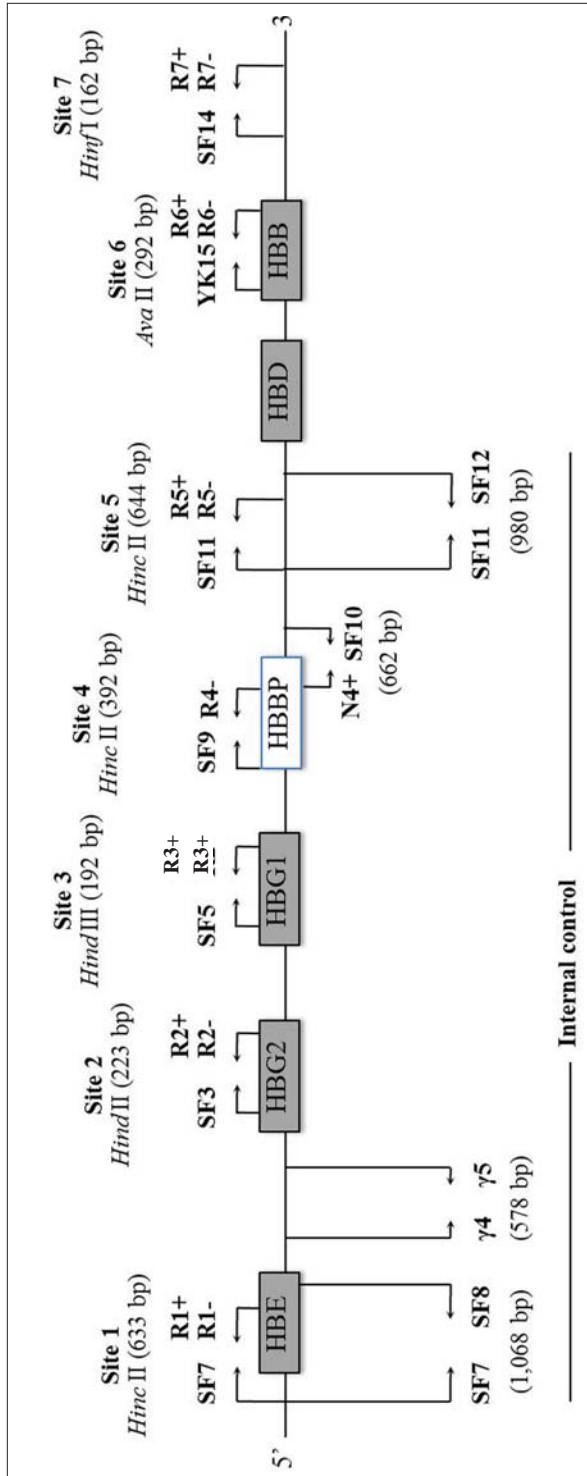| Parameters	Heterozygous β$^+$ -thalassemia	Homozygous β$^+$ -thalassemia
No	7	1
Origin	Thai	Japanese
RBC (x 10^{12}/l)	5.4 ± 0.8	4.3
Hb (g/dL)	14.5± 1.8	7.2
HCT (%)	42.9 ± 5.6	32.0
MCV (fl)	76.8 ± 7.5	74.4
MCH (pg)	24.5 ± 1.0	16.7
MCHC (g/L)	32.1± 2.2	22.5
Hb A$_2$ (%)	4.5 ± 0.7	5.9
Hb F (%)	0.9 ± 0.4	14.0
α-genotype	α / αα	αα / αα
β-genotype	β^A / β^{-31}	β^{-31}/ β^{-31}

Fig.1. The locations and orientations of primers used in allele specific PCR assays for 7 polymorphisms within β-globin gene cluster and the sizes of amplified fragments. Primers with + and − indicate allele specific primers for the presence and absence of polymorphic sites, respectively. PCRs are carried out in multiplex manners for polymorphic sites (1 and 2), (3 and 5) and (6 and 7) and a monoplex manner for site 4. HBE, HBG2, HBG1, HBBP, HBD and HBB are ε-, ^{G}γ-, ^{A}γ-, ψβ-, δ- and β-globin genes, respectively

Table 1: Allele specific and common primers used in allele specific PCR (ASPCR) for β-globin gene polymorphic analysis. + and – indicate primers for the presence and absence of polymorphic site, respectively. Size of each amplified fragment is indicated

| Polymorphism	Nucleotide change and location	Specific primer	Common primer	Size (bp)
Site 1: 5' εHinc II	A - insertion rs77526129	R1 + ; 5' TGCCTTAAAGGTGGTGACAGTT 3' R1 - ; 5' GCCTTAAAGGTGGTGACAGTG 3'	SF7 ; 5' GGCACATGGATCGAATTGAA 3'	633
Site 2: GγHind III	G → T rs2070972	R2 + ; 5' CCTCCAGATAACTACACACCA 3' R2 - ; 5' CCTCCAGATAACTACACACCC 3'	SF3 ; 5' GTTTGTGTGTGTGTGAGAGC 3'	223
Site 3: AγHind III	G → T rs28440105	R3 + ; 5' CCTCCAGATAACTACACACCA 3' R3- ; 5' CCTCCAGATAACTACACACCC 3'	SF5 ; 5' TTAACGTCTTCAGCCTACAA 3'	192
Site 4: ψβHinc II	G → A rs10128556	N4 + ; 5'TATGTTGGGGTAGTGAGTTGA3' R4 - ; 5' CCTTCTCTTACTTGCTATGC 3'	SF10 ; 5' CTCTTTTCTTGCAGGATTGC 3' SF9 ; 5' GGGAACAGAAGTTGAGATAG 3'	662 392
Site 5: ψβ 3'Hinc II	G → A rs968857	R5 + ; 5' AATGCATGACACATGCTTGT 3' R5- ; 5' TGCATGACACATGCTTGC 3'	SF11 ; 5'GCTCCATGAACAAACATTCC 3'	644
Site 6: βAva II	G → C rs10768683	R6 + ; 5' GGGAAAGAAAACATCAAGG 3' R6 - ; 5' GGGAAAGAAAACATCAAGC 3'	YK15 ; 5' TCTCTCTGCCTATTGGTCTA 3'	292
Site 7: β 3'Hinf I	T → A rs10837631	R7 + ; 5' GCAAGAGAACTGAGTGGAGT 3' R7 - ; 5' GCAAGAGAACTGAGTGGAGA 3'	SF14 ; 5' TCCCTCAGAAAAGGATTCAA 3'	162
		Internal control for sites 1 & 2	SF7 ; 5' GGCACATGGATCGAATTGAA 3' SF8 ; 5' ACCATGATGCCAGGCCTGAG 3'	1,068
		Internal control for sites 4 and 6 &7	γ4 ; 5' GGCCTAAAACCACAGAGT 3' γ5 ; 5' CCAGAAGCGAGTGTGTGGAA 3'	578
		Internal control for sites 3 & 5	SF11 ; 5' GCTCCATGAACAAACATTCC 3' SF12 ; 5' AAGGAGCACCCACTAGCTCA 3'	980
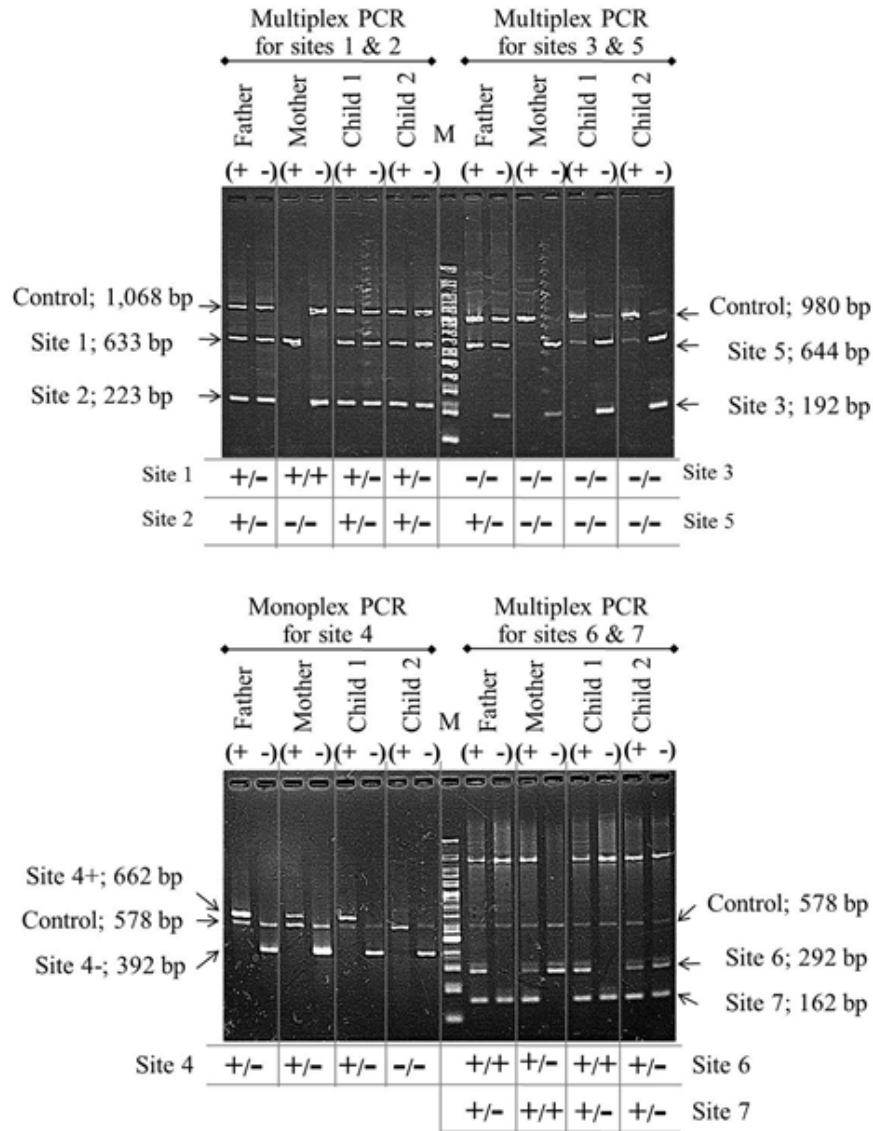
Fig. 2. Representative gel electrophoresis of allele specific PCR assays for β-globin gene polymorphic analysis in a family with 4 members including the father, the mother and the two children. (+ -) above the gel of each individual indicates allele specific PCR for the presence (+) and absence (-) of each polymorphic site, respectively.
Upper: Multiplex allele specific PCR for sites (1 and 2) and (3 and 5). The 1,068, 633 and 233 bp fragments are specific fragments for internal control, site 1 (ε-*Hinc* II) and site 2 (ᴳγ-*Hind* III), respectively. M represents the Gene Ruler 100 bp Plus DNA ladder. The 980, 644 and 192 bp on sites ᴳγ (3 and 5) are specific fragments for internal control, site 5 (3'ψβ-*Hinc* II), and site 3 (ᴬγ-*Hind* III), respectively. Interpretation of polymorphic analysis of each individual is indicated below the gel.
Lower: Monoplex allele specific PCR for site 4 and multiplex allele specific PCR for sites (6 and 7). The 662, 578 and 392 bp fragments are specific fragments for site 4 + (ψβ-*Hinc* II), internal control and site 4 - (ψβ-*Hinc* II), respectively. M represents the Gene Ruler 100 bp Plus DNA ladder. The 578, 292 and 162 bp fragments on a multiplex allele specific PCR for sites (6 and 7) are specific fragments for internal control, site 6 (β-*Ava* II) and site 7 (3' β-*Hinf* I), respectively. Interpretation of polymorphic analysis of each individual is indicated below the gel.

Representative gel electrophoresis of the DNA polymorphic analysis using new allele specific PCR assays developed are shown in Figure 2. The method allowed identification of each polymorphic site without restriction digestion. As shown in the figure, each polymorphism can be readily determined on the gel electrophoresis of the PCR product. The results obtained using these allele specific PCR assays matched one hundred percent with β-globin haplotyping using conventional PCR-restriction fragment length polymorphism assays (data not shown). These newly developed PCR assays were applied to the determination of β-globin gene haplotype linked to the -31 A-G mutation β⁺-thalassemia in Thai subjects. As shown in Table 3, it was found that all the seven Thai β⁺-thalassemia alleles with his mutation were associated with β-globin gene haplotype (+ - - - - - +), which is difference from that described for a Japanese haplotype (- + + - + + -) (Takihara et al. 1986).

DISCUSSION

The β⁺-thalassemia with -31 A-G mutation has been mainly described among the Japanese where it was associated with a β-globin gene haplotype (- + + - + + -). This mutation is the most common β-thalassemia allele observed in this population with a frequency of 16.3 percent. It has been thought that this mutation is unique to the Japanese and has arisen after Japan islands were separated from the Asian continent about 10,000 years ago (Hattori et al. 2003). This A-G mutation occurs at the second base of the TATA box within a highly conserved proximal promoter of β-globin gene leading to a two-fold decreased in transcriptional efficiency and a reduced interaction with TATA-binding protein (Takihara et al. 1986; Savinkova et al. 2013). Homozygous for this mutation was associated with a mild thalassemia intermedia phenotype (Hattori et al. 2003). Recent study in Thailand has unexpectedly noted on a high prevalence of

this mutation in the population being 7.8 percent of 528 β⁺-thalassemia genes examined (Yamsri et al. 2015). As for other β-thalassemia mutations, heterozygous for this mutation is associated with reduced mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values and elevated Hb A$_2$ (Table 2) (Saeung et al. 2012). Compound heterozygous of this mutation with Hb E has a mild thalassemia intermedia phenotype (Vathana et al. 2005). Since β⁺-thalassemia is common and heterogeneous among Thai population, identification of this mutation is therefore essential for genetic counseling and prenatal diagnosis (Yamsri et al. 2010; Fucharoen et al. 2007). In the laboratory this mutation is routinely identified using allele specific PCR or high resolution melting (HRM) assay (Prajantasen et al. 2015). However, because of a mild thalassemia intermedia phenotype, prenatal diagnosis is not usually offered for couple at risk of having fetus with homozygous or compound heterozygous with Hb E of this mutation.

Using allele specific PCR assays developed as shown in Figures 1 and 2, the researchers have now demonstrated for the first time a non-Japanese origin of the -31 A-G β⁺-thalassemia in Thailand. All seven β⁺-thalassemia alleles with -31 A-G mutation in Thai subjects are alternatively linked to haplotype (+ - - - - - +) (Table 3). This haplotype data in the study likely indicates a unique evolutionary origin of the β-31 A-G mutation in Thai population. This mutation has rarely been described in other populations. On the basis of difference in haplotype between Japanese and Thai and a relatively common allele in Thai population, this β-31 A-G mutation likely arose independently in the Thai population and represented another origin of this β⁺-thalassemia gene. Such phenomenon has also been observed for other hemoglobinopathies such as β⁺-thalassemia with -86 C-G mutation (He et al. 2015) and -90 C-T mutation (Prajantasen et al. 2014), β⁰-thalassemia among Yunnanese (Zhang et al.

Table 3: β-Globin gene haplotype associated with the -31 A-G β⁺-thalassemia in a Japanese and Thai subjects + And – indicate the presence and absence of a polymorphic site, respectively

Subject	HincII	Hind III	Hinc II	Ava II	Hinf I		No. of chromosome		Reference
	5'-ε	Gγ	Aγ	ψβ	ψβ-3'		β	β–3'	
Japanese	-	+	+	-	+	+	-	2	Takihara et al. 1986
Thai	+	-	-	-	-	-	+	7	This study

2015), (γδβ)⁰-thalassemia (Singha et al. 2015), Hb E (Fucharoen et al. 2002), Hb C (Sanchaisuriya et al. 2001), Hb Constant Spring (Singsanan et al, 2007) and Hb D-Los Angeles (Mahdavi et al. 2015). Study on the haplotypic heterogeneity of β-globin gene mutations in geographic regions and multiple populations should provide useful information on ethnic relationship and evolutionary view of human populations as well as for population genetics study of the hemoglobinpathy.

CONCLUSION

Study of genetic origin, examination of ethnic relationship, and linkage analysis of β-globin gene mutation are usually conducted on 7 restriction polymorphic sites in β-globin cluster using PCR-RFLP methodology. The method involves restriction digestion of DNA, which is considerably laborious, expensive and time-consuming. In contrast, β-globin haplotyping using allele specific PCR assays developed in this study is relatively simple and rapid, does not require restriction digestion of DNA and the result is readily obtained on gel electrophoresis of PCR product.

RECOMMENDATIONS

The assays have been used to demonstrate an independent origin of β+-thalassemia with -31 A-G mutation in Thai individuals and should prove useful in further applications in linkage analysis and population genetics study of β hemoglobinpopaphy.

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